the identities of the important acids, and consequently the paper chromatographic data shown in Tables IV and V are sufficient for confirmation of acetic, propionic, butyric, caproic, and capric.

Although the paper chromatographic data show formic acid to be present in substantial quantity, it was not detected on the gas chromatograms. Known formic acid solutions also gave no peak. Apparently either decomposition of formic acid takes place in the gas chromatograph, or else the acid combines with the liquid stationary phase (Carbowax 20M) and therefore never elutes from the column.

The acids in low concentrations, as indicated by the minor peaks 4, 5, 6, and 8, received only tentative identification by retention temperature coincidence on one column, although Table IV does show evidence for the presence of a 5-carbon acid. Peak 9 remains as an unknown.

At least seven components not previously identified as constituents of oranges or processed orange products have been reported. These newly identified compounds are *n*-propanol, isobutanol, *n*-butanol, isopentanol, terpinen-4-ol, *n*-decanol, and *n*-caproic acid. Also, 10 compounds previously tentatively identified (*10*) were confirmed.


### Acknowledgment

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**FEED ADDITIVE RESIDUES**

# Determination of Trace Amounts of Nitrofurazone in Milk


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**A method has been developed for quantitative determination of nitrofurazone in milk with a sensitivity of 0.01 p.p.m. The procedure is based on conversion of nitrofurazone to 5-nitrofurfuraldehyde phenylhydrazone, followed by extraction and concentration on a chromatographic column. Final estimation depends upon development of a blue color with Hyamine base.**


NITROFURAZONE is used for the treatment of mastitis in cattle. Methods previously available for determination of nitrofurazone residues in milk include those of Paar (*7*) and of Cox and Heotis (*5*). Paar's method determines nitrofurazone by direct photometric measurement at the 375 mμ maximum after precipitation of milk proteins with sodium tungstate and sulfuric acid. The method is very simple and suitable for levels of 1 p.p.m. or more. The limit of detection is 0.5 p.p.m. The method of Cox and Heotis involves conversion of nitrofurazone to 5-nitrofurfural phenylhydrazone with subsequent toluene extraction and chromatography, finally measuring the phenylhydrazone compound at 430 mμ. The procedure is sensitive to 0.25 p.p.m. Colorimetric estimation of the phenylhydrazone is also the basis of methods for determination of nitrofurazone in feeds (*1*), plasma (*4*), and tissues (*6*).

A method for determining nitrofurazone in milk sensitive to 0.01 p.p.m was needed to meet a Food and Drug Administration requirement. The procedure developed consists of converting any nitrofurazone present to 5-nitrofurfural phenylhydrazone followed by extraction in toluene as in the Cox and Heotis method (*5*). A sensitivity of 0.01 p.p.m. is attained by use of the following modifications. A large sample of milk, 120 ml., is used for each determination. Sodium chloride is added to prevent the formation of gel in the toluene extract. This permits recovery of a larger portion of extract. Column chromatography is used as a means of concentrating the phenylhydrazone compound. A blue color is developed; thus the wave-length of color measurement is out of the range of milk pigment interference.

### Materials and Methods

**Reagents and Instrument.** The reagents used include phenylhydrazine hydrochloride, 1% in water, freshly prepared for each set of samples; aluminum oxide, Merck chromatographic grade; hydroxide of Hyamine 10× (Rohm & Haas, Reg. Trade Mark), 1 molar solution in methanol as used in preparation of samples for scintillation counting (available from Packard Instrument Co., Lagrange, Ill.); crystalline nitrofurazone standard (Hess & Clark). A Beckman Model DU spectrophotometer was used for all absorbance measurements.

**Preparation of Standard.** Prepare the standard curve by assay of samples of control milk with known amounts of nitrofurazone added. Dissolve 100 mg. of pure nitrofurazone in 100 ml. of dimethylformamide. Dilute 1 ml. of this solution to 100 ml. with water. Each milliliter of this standard contains 10 μg. of nitrofurazone. Measure 200-ml. portions of control milk

**Table I. Absorbances at 530 mμ of Preparations from Control Milk Fortified with Graded Amounts of Nitrofurazone (NFZ)**

(Color developed with Hyamine hydroxide)

| Sample | Number of Samples | Mean Absorbance | Std. Dev. | Range |
|---|---|---|---|---|
| Control Milk | 18 | 0.007 | 0.003 | 0.002–0.012 |
| Milk + 0.01 p.p.m. NFZ | 12 | 0.016 | 0.003 | 0.010–0.021 |
| Milk + 0.02 p.p.m. NFZ | 5 | 0.024 | 0.003 | 0.020–0.027 |
| Milk + 0.05 p.p.m. NFZ | 5 | 0.049 | 0.003 | 0.045–0.052 |
| Milk + 0.10 p.p.m. NFZ | 4 | 0.087 | 0.006 | 0.079–0.091 |

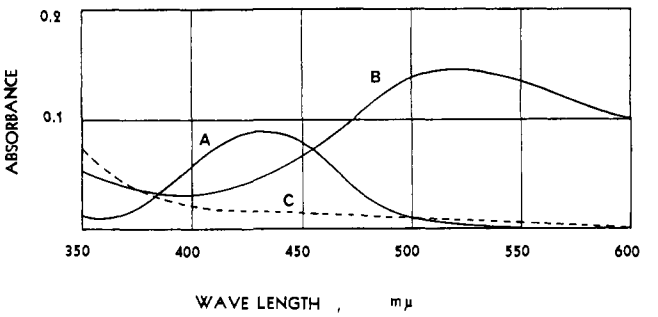


Figure 1. Absorbance curve for (A) 5-nitro-2-furfuraldehyde phenylhydrazone, 1 p.p.m. toluene-isopropanol (10:1); (B) same solution after addition of 1 drop of Hyamine; and (C) control milk eluate after addition of 1 drop of Hyamine

into flasks and add 0.0, 0.2, 0.4, 1.0, and 2.0 ml. of the standard nitrofurazone solution. Put these standard milk solutions, containing zero, 0.01, 0.02, 0.05, and 0.10 p.p.m. nitrofurazone, through the complete procedure. Protect nitrofuran solutions from sunlight or fluorescent lighting at all times to avoid decomposition.

**Procedure.** For milk containing less than 0.1 p.p.m. nitrofurazone, use a 120-ml. sample. Measure 30 ml. of the milk sample into each of four centrifuge tubes (borosilicate glass, 40 ml., heavy duty, with neck).

Pipet 0.5 ml. of phenylhydrazine hydrochloride 1% and 2 ml. of concentrated HCl into each tube. Shake, using polyethylene stoppers, then place the tubes in a rack in a constant temperature bath at 70° C. for 15 minutes. The bath is of such capacity that the water temperature is not appreciably lowered when the tubes are inserted. After exactly 15 minutes, remove the rack and cool the tubes in a cold water–chipped ice bath for 5 minutes. Pipet 8 ml. of toluene into each tube and add approximately 6 grams of granular sodium chloride. Stopper and shake vigorously for 1 minute. Centrifuge at 2000 r.p.m. for 10 minutes.

Decant the toluene layers from the four tubes and pool them in a single graduated cylinder. The semisolid layer beneath the toluene is quite firm and holds back the aqueous phase in the centrifuge tube. Between 25 to 29 ml. of toluene solution will be recovered depending upon the nature of the milk. Add a few crystals of anhydrous sodium sulfate to take up any water present.

The chromatographic tubes used are 300- × 10-mm. i.d. Insert a small plug of borosilicate glass wool. Add the adsorbent as a slurry of 2.7 grams Merck alumina in 10 ml. of toluene. Wash down the columns with additional toluene and top with another small plug of borosilicate glass wool.

Add 25 ml. of the toluene sample solution to the column. When the last of this has entered the adsorbent, add 15 ml. of toluene. The nitrofuran phenylhydrazone, if present, will be seen as a red band at the top of the column just below a yellow band of milk pigment. When the toluene has drained, add exactly 2 ml. of 10:1 toluene-isopropanol and again let all of the liquid pass into the adsorbent. At this point the red band should be midway down the column. Add 5 ml. of 10:1 toluene-isopropanol, and collect the eluate in a small test tube graduated at 5 ml. Transfer to a 1-cm. cell and read the absorbance at 530 mμ. This reading will normally be less than 0.005 and will be subtracted as a blank. Add 1 drop of Hyamine hydroxide solution. Immediately mix and again read the absorbance at 530 mμ.

By using the corrected absorbance read the amount of nitrofurazone present in the sample from the standard curve.

## Discussion

The conversion of nitrofurazone to the phenylhydrazone compound in the presence of organic materials is reproducible, but not complete. Time, temperature, acidity, and concentrations are interrelated factors affecting the color yield and must be kept constant. Optimum conditions were determined for the reaction in milk. Addition of more acid is required in milk than in water solution. For accuracy, therefore, standards must be prepared in control milk rather than in water. Absorbance values obtained with control milk and standards are shown in Table I. The absorbance values plotted against concentration fall on a straight line.

Extraction of the reaction mixture in milk with toluene alone yields a thick gel so that even after double centrifugation only a fraction of the toluene solution could be recovered. This problem was eliminated by the addition of granular salt. Excellent phase separation is then attained and the clear toluene solution can be almost completely recovered.

For chromatography of the toluene solution a comparison was made of dry-packed *vs.* slurry-packed alumina columns. Columns packed by slurrying the alumina in toluene were much more uniform and permitted faster flow rates. When the toluene extract from milk passes through the column, some of the milk pigments are eliminated with the toluene wash. The nitrofuran appears as a red zone just below the surface of the alumina, with a faint yellow pigment from the milk lying just above it. At 0.01 p.p.m. concentration in the milk, the red nitrofuran phenylhydrazone band is just visible in comparison with a control column. At 0.05 p.p.m. or more, the red band is clearly evident.

Various solvent combinations were tested for separation of the red and yellow bands. None was found to accomplish a separation with a reasonable length of column and amount of solvent. Since the faint yellow band contributes no interference at 530 mμ, it appeared more advantageous to elute the red zone in the smallest possible volume without attempting partition of absorbed pigments. Alcohol mixtures with toluene were very satisfactory. A mixture of 10 parts toluene with 1 part isopropanol was selected as eluting with the smallest spread. By following the chromatography procedure as described, the nitrofuran is consistently isolated in an eluate volume of 5 ml., even when the red zone is scarcely visible.

The reaction of nitrofurans with alcoholic KOH has been used for determination of these compounds in poultry feeds (*2*, *3*). The exact mechanism of the color production in these cases is unknown although characteristic color in alkaline solvent solutions is common to very many aromatic nitro compounds, and is explained by resonance mechanisms (*8*). Addition of alcoholic KOH to eluates from fortified milk samples produced a blue color, but was not satisfactory because of fading and tendency of the solutions to become cloudy. The strong organic base Hyamine hydroxide gave clear blue solutions with excellent color stability. Standards exhibited approximately 3%

loss on standing 1 hour. At the absorbance maximum, 530 mμ, interference from milk pigments is much less than at 430 mμ. A further advantage is that the specific absorbance of the blue color at 530 mμ is somewhat greater than the corresponding nitrofuran absorbance at 430 mμ. The absorbance curves for each of these, as recorded with a Beckman DK spectrophotometer, are shown in Figure 1.

This method has been applied to the milk from cows treated with a commercial nitrofurazone preparation according to label directions. The results will be reported in a separate publication.

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**FEED ADDITIVE RESIDUES**

# A Colorimetric Procedure for the Microdetermination of Sulfonamides in Animal Tissues


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A colorimetric procedure for the determination of microgram quantities of *p*-aminobenzenesulfonamides is presented. The sulfonamide is adsorbed, from an acid medium, on a strong cationic exchange resin in the hydrogen ion form. The column is then washed with acid and water to remove interfering compounds and the sulfonamide subsequently eluted with ammonium hydroxide. The Bratton-Marshall colorimetric procedure is used for the quantitative measurement of the sulfonamide. The average recovery of two sulfonamides from swine, calf, chicken, and turkey tissues, as well as bovine milk, ranged from 71.9 to 96.5%. The control values obtained on the analysis of unmedicated tissues (blood, muscle, liver, kidney, and fat) ranged from 0.050 to 0.181 p.p.m. of apparent sulfonamide. Thirteen different sulfonamide standards were successfully carried through this procedure. A minimum of 2.5 μg. of the sulfonamide can be accurately determined.


THE SULFONAMIDES, because of their bacteriostatic properties, have recently been proposed for use as animal feed supplements in combination with antibiotics as a prophylactic aid in increasing the growth rate in swine, improving feed efficiency, and reducing losses associated with bacterial diseases (*2*).

Because of this new aspect of sulfonamide therapy and the low treatment levels involved, interest developed in a highly sensitive tissue residue analysis procedure. This necessitated the development of a highly specific cleanup procedure which would eliminate naturally occurring interfering compounds found in the tissues and other chemotherapeutic agents present.

Existing procedures using the Bratton-Marshall colorimetric reaction for the determination of primary aromatic amines (*3*) employed no cleanup other than protein precipitation and dilution. These procedures were inadequate because of the low levels of sulfonamide present and the high control values obtained from the analysis of unmedicated tissue.

A method is presented whereby the sulfonamide is extracted from the sample with a mixture of acetone and carbon tetrachloride and then partitioned into 1*N* HCl. The aqueous acid solution is subsequently passed through a strong cationic exchange resin in the hydrogen ion form, resulting in adsorption of the sulfonamide on the resin. After being washed free of interferences with water, the compound is eluted with concentrated $NH_4OH$, acidified, heated to convert any acetyl compound to the free acid, and quantitatively measured by the Bratton-Marshall colorimetric procedure (*1*).

This technique has been successfully used to separate and distinguish aminobenzenesulfonamides from procaine penicillin, which also reacts to the Bratton-Marshall test and is commonly used as a feed supplement and in animal medication. The method is capable of quantitatively determining as little as 2.5 μg. of the sulfonamide.

### Experimental

**Reagents.** Sodium nitrite, 0.1%. Ammonium sulfamate, 0.5%. *N*-(1-naphthyl)ethylenediamine dihydrochloride, 0.1%. Aqueous solutions of these three reagents should be prepared fresh daily.

Trichloroacetic acid, aqueous 50% solution.

Dowex 50W-X2 (H+) ion exchange resin, 50- to 100-mesh (J. T. Baker Co.). Transfer 1 pound of dry resin (as shipped) to a 2000-ml. glass funnel with a fused, coarse fritted disk, and add 500 ml. of 1*N* NaOH (C. P. reagent). Stir with a glass rod until well mixed and allow the mixture to filter by gravity for 5 minutes. Apply suction and remove the NaOH solution until approximately 5 mm. of liquid remains above the resin bed. At no time should the resin be allowed to become dry. Repeat the above treatment until at least 2 liters of 1*N* NaOH have been used or until the filtrate is colorless. Wash the resin with distilled water, filtering with suction, until the effluent is the same pH as the distilled water. Regenerate the resin by the addition of 500 ml. of 1*N* HCl to the funnel. Mix well with a glass rod and allow the slurry to filter by